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JOURNAL OF MEMBRANE BIOLOGY, (1999 Mar 1) 168 (1) 77-89.

Proc. Natl. Acad. Sci. U. S. A. (1993), 90(10), 4591-5

International Congress Series (1995), 1066(Atherosclerosis X), 838-43

GENOMICS, (1994 Jan 15) 19 (2) 350-7.

JOURNAL OF CLINICAL INVESTIGATION, (1994 Aug) 94 (2) 885-91.

FASEB JOURNAL, (1995 Jul) 9 (10) 874-82.

JAPANESE HEART JOURNAL, (1996 Jan) 37 (1) 19-32.

ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY, (1998  
Mar) 18 (3) 349-55.

PHYSIOLOGICAL GENOMICS, (2000 Sep 8) 3 (3) 163-73.

HYPERTENSION, (1996 Dec) 28 (6) 1132-7.

Circulation Research (2002), 90(12), 1234-1236

Thank-You!

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## Regulation of PDGF-B in Endothelial Cells Exposed to Cyclic Strain

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**Abstract**—The present study was designed to examine the regulation by cyclic strain of endothelial cell (EC) platelet-derived growth factor-B chain (PDGF-B) expression. We demonstrate in this study that bovine aortic ECs subjected to 10% (but not 6%) average strain resulted in a 2.6-fold increase in PDGF-B steady state mRNA and immunoreactive protein. Nuclear runoff transcription assays confirmed the induction of PDGF-B transcripts. To address the regulation of PDGF-B gene expression by cyclic strain, we transfected bovine aortic ECs with a construct containing 450 bp of human PDGF-B promoter sequence coupled to chloramphenicol acetyltransferase (CAT), and found that subjecting these cells to 10% average strain resulted in a twofold increase in CAT activity by 4 hours. Analysis of nested 5' deletions of the promoter transfected into ECs demonstrated a 55% drop-off in activity between position -313 and -153, with no induction of activity with the -101-bp minimal promoter. Since a shear stress response element (SSRE) is located at position -125, we tested the hypothesis that the SSRE site was necessary and/or sufficient for induction of PDGF-B activity with strain. Electromobility shift assays revealed that nuclear proteins from ECs exposed to strain for short intervals (30 minutes) bound to the PDGF-B SSRE. However, transfection of ECs with hybrid promoter constructs containing the SV40 sequence promoter downstream of the SSRE or the -153 PDGF-B promoter sequence bearing a mutation in the SSRE demonstrated that the SSRE was not necessary for inducible reporter gene expression in ECs exposed to cyclic strain. (*Arterioscler Thromb Vasc Biol.* 1998;18:349-355.)

**Key Words:** endothelium ■ hemodynamics ■ PDGF-B ■ gene expression

PDGF-B is a dimeric protein composed of two similar but structurally distinct polypeptide chains, denoted A and B, and is expressed by a variety of cell types.<sup>1-5</sup> In vitro, ECs basally express mRNA for both subunits, with some variability depending on the cell source. Several studies indicate that these subunits are induced in response to a myriad of chemical mediators and physical forces. Recent reports demonstrate that exposure to laminar shear stress induces a 10-fold increase in PDGF-A chain mRNA<sup>6</sup> and a twofold to threefold increase in B chain mRNA expression<sup>7-9</sup> in both human umbilical vein and bovine aortic ECs. An SSRE was defined using 5' deletions of the human PDGF-B promoter coupled to a CAT reporter gene in transient transfection analyses of bovine aortic ECs. This promoter sequence was found to be both necessary and sufficient for increased PDGF-B promoter-dependent expression in cultured ECs exposed to laminar shear stress.<sup>9</sup>

ECs in vivo are also subjected to the repetitive distention of the blood vessel wall during the cardiac cycle. It is not clearly known, however, whether ECs exposed to this particular physical force sense and respond in an analogous manner as with exposure to shear stress. The objective of this study was to determine whether exposing cultured ECs to cyclic strain could regulate the expression of PDGF-B.

## Methods

### Cell Culture

Bovine aortic ECs were obtained by gently scraping the intimal surface of bovine thoracic aorta and were maintained in Dulbecco's modified Eagle's medium/F-12 (1:1) mixture (with 50 nmol/L HEPES buffer, pH 7.2, 10 mmol/L glutamine and NaHCO<sub>3</sub>) supplemented with 10% heat-inactivated calf serum, 5 µg/mL deoxycytidine/thymidine, antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL) and amphotericin B (250 ng/mL). Bovine aortic ECs were identified by their typical morphological and chemical characteristics, including growth of closely opposed polygonal confluent monolayers, maintenance of density-dependent inhibition after serial passage, positive indirect immunofluorescence staining for Factor VIII antigen, and the uptake of di-I-acetylated LDL.

### Experimental Protocol

The strain unit (Flexcell, Flexercell Corp) consisted of a vacuum manifold with recessed ports and has been described in detail previously.<sup>10,12</sup> Bovine aortic ECs were cultured on plates with bottoms made of flexible silicone elastomer covered with collagen type I (Flex I, Flexercell Corp). The plates were centered over ports on the vacuum manifold. A vacuum line was connected to regulator solenoid valves that were in turn controlled by a computer with a timer program. Thus, the design of the strain unit allows for changes in amplitude, frequency, and duration of the applied strain for each given experiment.

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### Selected Abbreviations and Acronyms

CAT	= chloramphenicol acetyltransferase
EC	= endothelial cell
EMSA	= electromobility shift assay
PDGF-B	= platelet-derived growth factor-B
SSRE	= shear stress response element

For these experiments, bovine aortic ECs were seeded onto Flex 1 stretch plates and grown to confluence. The membranes were subjected to deformation with 37.5 mm Hg or 150 mm Hg of vacuum at a frequency of 60 cycles per minute (0.5 seconds of deformation alternating with 0.5 seconds of relaxation) for up to 24 hours. A vacuum of 150 mm Hg produces a deformation pattern ranging from 0% at the center of the membrane to 24% at the periphery (average strain, 10%). A 37.5-mm Hg vacuum results in a deformation ranging from 0% in the center to a maximum of 10% in the periphery (average strain, 6%).<sup>10,11-15</sup> For the sake of simplicity, the two experimental groups are referred to as the 6% and 10% average-strain groups.

The highest strain is found in a region 9.5 mm from the center of a 25-mm-diameter well. Thus, in the SSRE EMSA described below, we took advantage of the heterogeneous strain gradient by using a fence to selectively seed cells either in the central low-strain region of the membrane or in the peripheral high-strain region.<sup>16-18</sup> The fence was removed after 24 hours and the selectively seeded plates were then subjected to a strain regimen of 150 mm Hg of vacuum at 60 cycles per minute. The cells seeded in the periphery experienced 7% to 24% strain, whereas the cells seeded at the center experienced <7% strain, with the majority of cells exposed to minimal strain.

### Northern Blot Analysis for PDGF-B

Total cytosolic RNA from stationary (control) ECs and from ECs that had been stretched for up to 24 hours was isolated by the guanidinium isothiocyanate method with phenol extractions.<sup>19</sup> RNA (10 to 15  $\mu$ g) was electrophoresed through a 1% agarose/1.1% formaldehyde gel, transferred to a nylon membrane (Zeta Bind, American Bioanalytical), and immobilized by ultraviolet irradiation. Hybridization was then performed with a random-primed, [ $\alpha$ -<sup>32</sup>P]dCTP-labeled, full-length cDNA probe coding for human PDGF-B (detects 3.4 kb mRNA) or human GAPDH (detects 1.2 kb mRNA) as previously described.<sup>9</sup> Autoradiography was carried out for 1 to 3 days using Kodak X-Omat AR-5 and Kodak XRP1 film and intensifying screens. Optical densities of hybridization signals on x-ray films were measured by densitometry (Visage 2000 Gel Analyzer, Bioimage) for quantification of steady state mRNA levels.

### Nuclear Runoff Transcription Assays

Nuclear transcription assays were performed,<sup>12</sup> with slight modifications of the procedure described by Greenberg and Ziff.<sup>20</sup> To isolate nuclei, stationary ECs and ECs that had been exposed to 10% average strain for 4 hours were lysed in buffer containing 10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl<sub>2</sub>, and 0.5% Nonidet P-40 and the nuclei recovered by centrifugation at 500g for 5 minutes at 4°C. The nuclei were then resuspended at 26°C for 30 minutes in runoff buffer (35% glycerol, 10 mmol/L Tris-HCl, pH 8.0, 5 mmol/L MgCl<sub>2</sub>, 80 mmol/L KCl, 0.1 mmol/L EDTA, 0.5 mmol/L DTT, 0.8 U RNasin, 4 mmol/L dATP, 4 mmol/L dGTP, 4 mmol/L dCTP) and 200  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]UTP (3000 Ci/mmol, Amersham). The nuclei were next digested with 10  $\mu$ g DNase 1 (RNase free) at 26°C for 5 minutes followed by incubation with 10 to 20 mg of proteinase K in buffer containing 5% SDS, 50 mmol/L EDTA, and 100 mmol/L Tris-HCl, pH 8.0, for 30 minutes at 37°C. Nascent elongated transcripts of radiolabelled RNA molecules were extracted by the guanidinium isothiocyanate method, precipitated with isopropanol, and dissolved in buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 1 mmol/L EDTA.

The level of incorporation of radioisotope was quantified in a beta-counter and a volume containing equal counts (for controls and for cells exposed to cyclic strain) was then hybridized over 24 hours at

65°C with membranes onto which linearized and alkaline-denatured PDGF-B and GAPDH probes had been immobilized by a slot-blot apparatus and UV radiation.

The membranes were then washed twice with 2X SSC at room temperature for 15 minutes followed by 1X SSC at 65°C for 15 minutes. Autoradiography was carried out for 1 to 3 days using Kodak X-Omat AR-5 and Kodak XRP1 film and intensifying screens.

### Immunohistochemical Staining for PDGF-B

Bovine aortic ECs exposed to 6% and 10% average strain for 24 hours at 60 cycles per minute were stained with the bovine PDGF-B antibody. In brief, cells were first fixed in 1% paraformaldehyde/0.1 mol/L borate buffer, blocked with normal horse serum 1:200, and then the bovine PDGF-B antibody (isotype IgG2a, 1:2000) was added for 1 hour. Binding of antibody was detected by the avidin-biotin, horseradish-peroxidase method with 0.05% 3,3'-diaminobenzidine tetrachloride in 0.05 mol/L Tris as the substrate. Specificity of the staining was assessed by incubating the cells to nonspecific mouse IgG2a or to the biotinylated secondary antibody only.

### Transfection Procedures and CAT Assay

The PDGF-B promoter-CAT reporter gene plasmids, and a series of 5'-deletion mutants encompassing the regions of interest, have been previously described.<sup>9</sup> A series of SSRE hybrid promoters have also been constructed by subcloning oligonucleotide inserts (the SSRE, GAGACC, or a nonsense sequence, C1C1CA) into the blunt-ended *Bgl* II site of an SV40-based enhancerless promoter controlling a CAT reporter gene (Promega).<sup>21</sup> In addition, site-directed mutants of the SSRE in the -153-bp construct had also been prepared by subcloning a polymerase chain reaction fragment bearing a block mutation in the SSRE.<sup>21</sup>

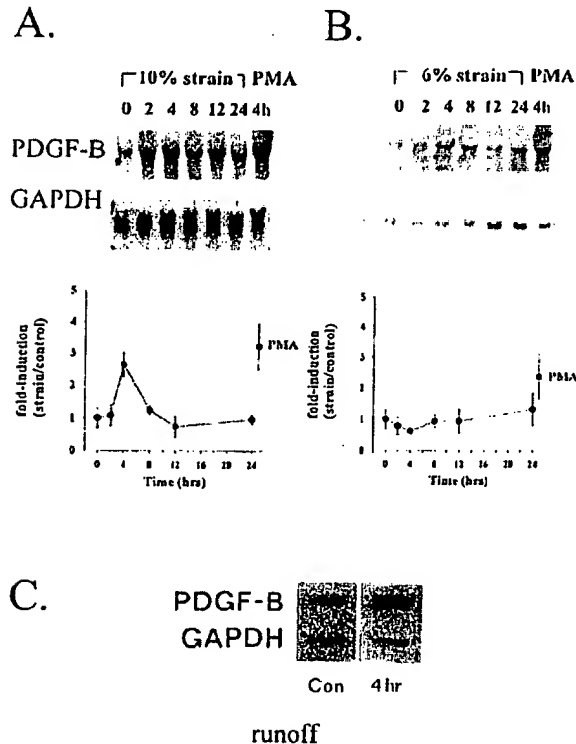
Cotransfection of ECs with the PDGF-B gene promoter-CAT gene constructs and a cytomegalovirus promoter *lac z* gene construct were performed, employing the calcium phosphate precipitation method, incorporating a glycerol shock.<sup>22</sup> Cell viability was maintained throughout the incubation period, and transfected cells showed typical responses to exposure to cyclic strain (eg, shape change and axial alignment with 60 cycles per minute, 24% strain). The transfected cells were cultured on Flex 1 plates. After exposure to cyclic strain, cells were harvested, and relative changes in CAT activity were quantified by determining the percentage of [<sup>14</sup>C]chloramphenicol converted to its acetylated products by liquid scintillation counting.<sup>22</sup> Transfection efficiency was evaluated by measurement of  $\beta$ -galactosidase.<sup>22</sup> Fold induction was calculated as the PDGF-B value divided by the GAPDH value for the different regimens divided by control, static conditions.

### EMSA of SSRE in Nuclear Protein Extract of ECs Subjected to Cyclic Strain

Double-stranded oligomers containing three repeating units of GAGACC (SSRE) were end labeled with [<sup>32</sup>P]ATP by T4 polynucleotide kinase and purified by Sepharose G-50 chromatography spin column twice. Nuclear protein extracts were prepared from 3  $\times$  10<sup>6</sup> ECs under static conditions or subjected to cyclic strain.<sup>23-25</sup> The nuclear protein extract was incubated with incubation buffer at 4°C for 15 minutes to minimize nonspecific protein/DNA interactions, and specific competition experiments were performed by adding unlabeled oligonucleotide to the control tubes at the same time. Labeled oligonucleotide was then added and left at room temperature for 20 minutes. The protein/DNA mixtures were applied to a 6% nondenaturing polyacrylamide gel in 0.25X Tris borate buffer (TBE) buffer and subjected to electrophoresis at 12 V/cm for 2 hours. The gel was dried and exposed to XRP film with an intensifying screen at 70°C. Competition studies were performed by adding molar excess quantities of unlabeled oligonucleotide 10 minutes before the addition of radiolabeled oligonucleotide.

### Statistical Analysis

Results are presented as mean  $\pm$  SE. Analysis of variance with post hoc testing or the Student's paired *t* test were used when appropriate to



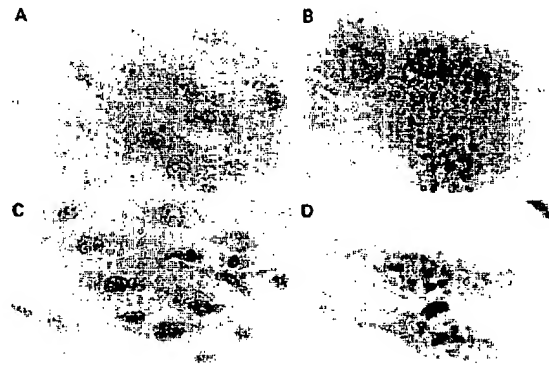
**Figure 1.** PDGF-B expression in ECs exposed to either 10% (A) or 6% (B) average strain at 60 cycles per minute. The top panels are typical Northern blots of ECs, demonstrating PDGF-B expression after 2, 4, 8, 12, or 24 hours of cyclic strain or after exposure to 25 ng/mL phorbol 12-myristate 13-acetate (PMA) for 4 hours. GAPDH is the constitutive control for loading. The graphs represent the average fold induction calculated from the densitometry values. The fold of induction is the PDGF-B value divided by the GAPDH value for the different regimens divided by control, static conditions. See "Methods" for details. Nuclear runoff study (C) demonstrates that PDGF-B transcript levels increased significantly in response to cyclic strain, whereas GAPDH transcript levels were unaffected. Con indicates control.

determine the significance of differences between means (Systat). A value of  $P < .05$  was considered significant.

## Results

### PDGF-B Chain Transcription and Protein Synthesis Are Increased in Cultured ECs Exposed to High But Not Low Cyclic Strain

Laminar shear stress applied to cultured ECs has been shown to increase the expression of PDGF-B.<sup>6-9</sup> Fig 1A shows a typical Northern blot of RNA extracted from ECs subjected to 10% average strain at 60 cycles per minute. Exposure of ECs to cyclic strain for 4 hours increased endogenous PDGF-B transcripts compared with stationary controls. Densitometric analysis of PDGF-B gene expression compared with GAPDH expression showed a PDGF-B/GAPDH ratio of  $2.6 \pm 0.4$  ( $n=4$ ,  $P < .01$ ) at 4 hours, which returned to control levels by 24 hours in bovine aortic ECs exposed to strain. To determine whether this increase in PDGF-B gene expression was dependent on the amplitude of strain, ECs were exposed to 6% average strain at 60 cycles per minute for various times. Fig 1B demonstrates that this level of strain did not induce the expression of the PDGF-B gene to any significant effect ( $n=4$ ).



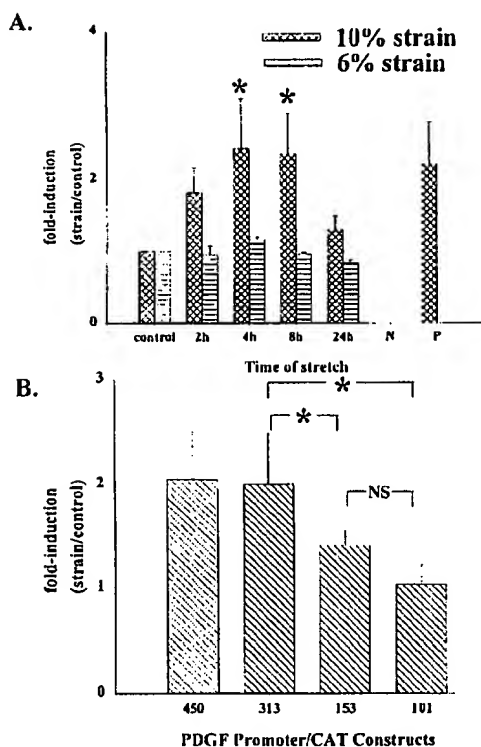
**Figure 2.** Immunocytochemical staining with PDGF-B antibody of ECs grown on membranes subjected to 150 mm Hg vacuum deformation at 60 cycles per minute for 24 hours (see "Methods" for details). A and C demonstrate the differential staining pattern across the membrane. ECs at the periphery (bottom) are elongated and aligned perpendicular to the force vector and show a more robust staining compared with ECs at the center of the membrane (top). B and D are high-power magnification ( $\times 400$ ) of individual cells in the different regions demonstrating the punctate staining.

Fig 1C is a representative nuclear runoff transcription assay. There was significant induction of new PDGF-B transcripts in nuclei isolated from ECs exposed to 4 hours of 10% average strain compared with nuclei from control stationary cells. The increase in PDGF-B transcription ( $2.9 \pm 0.5$ ,  $n=4$ ,  $P < .01$ ) was specific, since only minimal induction of GAPDH transcripts ( $1.1 \pm 0.3$ ,  $n=4$ ) was observed with cyclic strain.

In this model of cyclic strain, the strain pattern across the stretch membrane is inhomogeneous. Cells seeded in the periphery of the membrane experience maximum strain (24% at 150 mm Hg of vacuum deformation), while cells at the very center of the membrane experience minimum strain (0%).<sup>14</sup> To examine whether differential patterns of PDGF-B protein expression occurred in areas of high versus low strain, PDGF-B was localized by immunohistochemical staining. Fig 2 shows that bovine aortic ECs at the periphery of the membrane (7% to 24% strain) showed more intense staining after 8 hours of exposure to cyclic strain compared with bovine aortic ECs at the center of the membrane (0% to 7% strain). High-power magnification reveals perinuclear distribution of punctate PDGF-B staining, which appears to be arranged around intracellular vacuoles. Specificity was confirmed by the lack of staining when bovine aortic ECs were exposed to nonspecific mouse IgG2a and biotinylated secondary antibody (horse anti-mouse, data not shown).

### Cyclic Strain Can Directly Influence PDGF-B Transcription

ECs were transfected with a 450-bp fragment of PDGF-B upstream promoter coupled to CAT gene and subjected to 60 cycles per minute, 6% or 10% average strain for 0 (control, stationary), 2, 4, 8, or 24 hours. As seen in Fig 3A, in ECs exposed to 10% average strain, there was a 2.2-fold induction of PDGF-B promoter activity at 4 and 8 hours, which started to decline by 24 hours. In contrast, there was no significant increase in activity in ECs exposed to 6% average strain at any time. The pattern was similar to that previously reported with



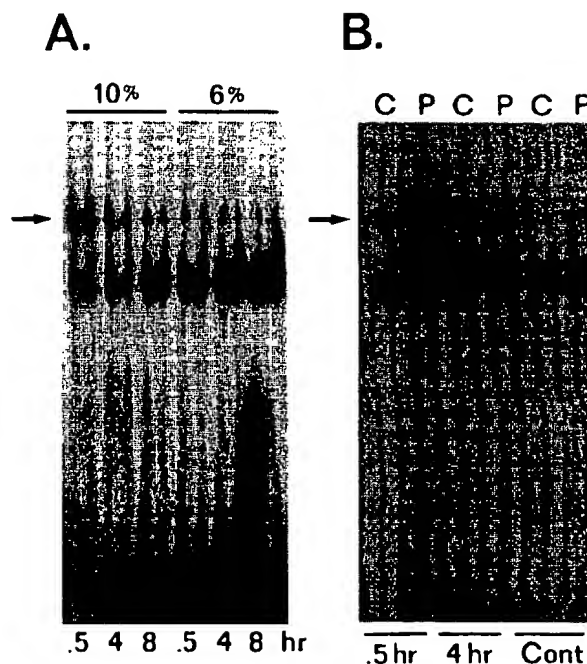
**Figure 3.** A, Fold induction of activity of a 450-bp human PDGF-B promoter fused to a CAT reporter gene transfected into ECs that were subjected to up to 24 hours of 6% or 10% average strain at 60 cycles per minute and then analyzed for CAT activity. The promoterless CAT vector (N) is a negative control, and a cytomegalovirus-driven CAT construct (P) represents a positive control. The folds of induction are the reporter CAT activity (normalized for transfection efficiency) in the experimental cells (strain) compared with those in static (control) cells. The mean of 4 to 6 separate experiments is given. \* $P < .05$ . B, Fold induction of CAT activity generated from PDGF-B promoter deletion mutants fused to a CAT reporter gene transfected into ECs that were subjected to 4 hours of 10% average strain at 60 cycles per minute. The lengths of the 5'-flanking sequences of the PDGF-B promoter fused to the CAT reporter gene are shown on the x axis. The folds of induction are the reporter CAT activity (normalized for transfection efficiency) in the experimental cells (strain) compared with those in static (control) cells. The mean of 4 to 10 separate experiments is given. \* $P < .05$ . NS indicates not significant.

shear stress," although the fold-stimulation signals were not as dramatic.

A series of constructs bearing 5' deletions of the PDGF-B promoter was transfected into ECs and subjected to 4 hours of 10% average strain. Comparison of promoter activity indicates that there was a 55% diminution in activity between the -313 and -153 position of the promoter and complete lack of strain-induced activity with the -101 promoter construct (Fig 3B).

#### Cyclic Strain Induces Nuclear Proteins That Bind to SSRE

We have previously identified an SSRE necessary for the increased PDGF-B production by ECs in response to fluid shear stress.<sup>9</sup> We performed EMSA using the SSRE oligonucleotide and nuclear extracts from bovine aortic ECs exposed

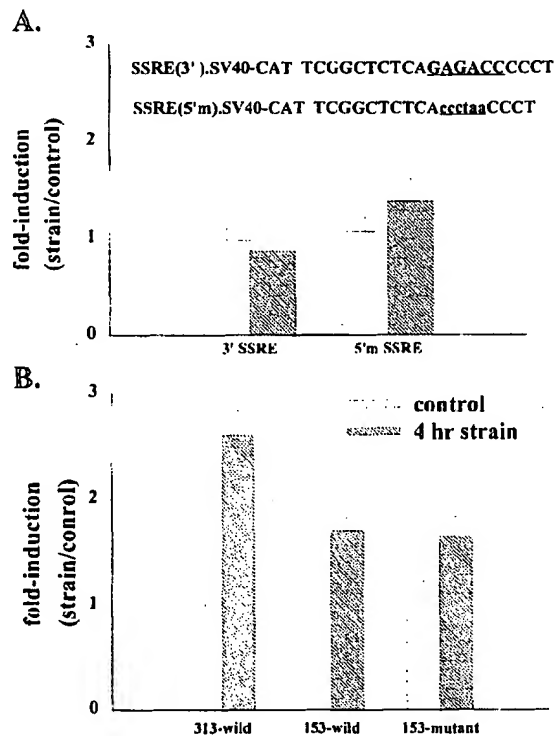


**Figure 4.** EMSA demonstrating binding to the SSRE oligomer. A, ECs were subjected to either 10% or 6% average strain for 0.5, 4, or 8 hours and the nuclear extracts subjected to EMSA with the SSRE oligonucleotide. The arrow points to the SSRE binding. B, Nuclear extracts from ECs located in the center (C) or periphery (P) of membranes that were deformed with 150 mm Hg vacuum at 60 cycles per minute for 0 (control, cont), 0.5, or 4 hours were subjected to EMSA with the SSRE oligonucleotide. Maximal induction of activity occurred within 30 minutes of exposure to cyclic strain.

to cyclic strain. As seen in Fig 4A, nuclear proteins binding the SSRE (arrow) were elevated in bovine aortic ECs exposed to cyclic strain for 30 minutes. These kinetics differed from those reported with shear stress, which were sustained for at least 4 hours after the onset of flow. The activation of these binding proteins is specific to ECs, since EMSA of nuclear extracts from cultured bovine aortic smooth muscle cells subjected to the same strain protocol failed to demonstrate SSRE binding (data not shown).

The strain dependence of this response can be seen in Fig 4B, which represents an EMSA using nuclear extracts obtained from ECs grown in the center or periphery of membranes that were deformed with 150 mm Hg vacuum. SSRE binding was detected when extracts from high-strain regions (periphery, 7% to 24% strain) were used, but not from low-strain regions (center, 0% to 7% strain; see Fig 4B). No SSRE binding was detected using nuclear extracts from ECs in the periphery or center of membranes deformed with 32.5 mm Hg vacuum (0% to 11% strain). SSRE binding was confirmed with cold competition assays (data not shown).

To determine whether the SSRE could mediate the induction of genes in ECs exposed to cyclic strain, the cells were transfected with SSRE- (GAGACC) or non-SSRE- (CTCTCA) containing hybrid promoters and subjected to 4 hours of cyclic strain. Fig 5A shows that there was no significant activation of either promoter. In fact, the non-SSRE hybrid showed a mild induction, which is consistent



**Figure 5.** Functional significance of the SSRE binding sites in the PDGF-B promoter. **A.** Hybrid promoters containing the core binding sequence of the SSRE (GAGACC) or an irrelevant sequence (ccctaa) linked to the CAT reporter gene were transfected into ECs that were subjected to cyclic strain for 4 hours. **B.** A mutant of the PDGF-B promoter fragment containing -153 bp of the upstream sequence fused to the CAT reporter gene was used and compared with the wild type -153- and -313-bp promoter fragment. SSRE-mutant represents a construct with a scrambled substitution of the SSRE sequence. The folds of induction are the reporter CAT activity (normalized for transfection efficiency) in ECs that were subjected to 4 hours of 10% average strain at 60 cycles per minute (strain) compared with those in static (control) cells. The mean of 3 separate experiments is given.

with that seen with exposure of the transfected ECs to laminar shear stress.<sup>27</sup> This result demonstrates that the SSRE element is not sufficient to confer cyclic-strain responsiveness. Furthermore, exposure of ECs transfected with the -153 promoter construct containing a site-directed mutation of the SSRE to cyclic strain resulted in activation of this promoter to the same extent as its wild-type counterpart (Fig 5B). These findings suggest that the SSRE site is not required for strain-induced PDGF-B promoter-dependent expression.

### Discussion

In vivo measurements in patients and animals and in vitro models simulating the major geometric features of blood vessels indicate that there is 5% to 6% wall excursion at peak systole under normal physiological conditions, which can be as high as 10% under hypertensive conditions,<sup>27-29</sup> thus exposing the endothelial lining to both shear stress and cyclic strain. Studies of cultured ECs exposed to biomechanical forces have shown that endothelial function can be influenced by these forces. We have demonstrated that cyclic strain alters various

parameters of EC function, such as the production of vasoactive substances.<sup>6,18,30-32</sup> In addition, cyclic strain increases EC proliferation and alters endothelial morphology (orientation of the long axis of the cell perpendicular to the axis of the strain)<sup>11,12,17,33</sup> and migration.<sup>34,35</sup> Likewise, several laboratories have demonstrated that shear stress can also affect EC morphology and their elaboration of vasoactive substances.<sup>6-8,36-39</sup> Thus, both cyclic strain and shear stress are capable of regulating EC function in vitro.

Our present studies demonstrate that cyclic strain, like shear stress, can increase PDGF-B gene expression in bovine aortic ECs. Northern blot analysis indicates that cyclic strain enhances endogenous PDGF-B gene expression in ECs in a time- and amplitude-dependent manner (Fig 1A and 1B). An average strain of 10% induced a rapid and robust increase in PDGF-B gene expression compared with 6% average strain. The differential effect of strain was confirmed by the presence of new PDGF-B transcripts after 4 hours of exposure of ECs to 10% average strain compared with the minimal induction of GAPDH (Fig 1C). This strain-amplitude dependence was also supported by greater PDGF-B immunostaining on ECs located in the high-strain membrane periphery (7% to 24% strain) relative to ECs attached to the low-strain center region (0% to 7% strain, Fig 2). Transient transfection analysis with a PDGF-B promoter-reporter construct extending 450 bp upstream of the TATA box are entirely consistent with these findings (Fig 3A). These results comprise the first observation that cyclic strain regulates the expression of PDGF-B. Our data are complementary to previous reports that shear stress increases PDGF-B gene expression and protein synthesis<sup>40</sup> and are consistent with in vivo investigations showing that PDGF-B levels are increased in areas of altered fluid dynamics. This is also the first demonstration that the amplitude of strain can influence PDGF-B gene and protein expression. Taken together, our studies and the findings of others suggest that PDGF-B production can be regulated by changes in pulsatile flow. Furthermore, strain patterns at bifurcations and branches have been demonstrated by finite analysis.<sup>41</sup> The greatest amount of wall strain appears to be at the toe and heel of a bifurcation. This observation may have clinical implications, as these sites are the most common for the development of atherosclerotic plaques and PDGF-B production is clearly enhanced in these regions.<sup>41,42</sup>

The differential expression of the PDGF-B gene in ECs exposed to varying levels of strain are consistent with previous studies that have demonstrated changes in EC proliferation,<sup>17</sup> tissue plasminogen activator expression,<sup>16</sup> and nitric oxide synthase levels<sup>18,30</sup> in response to different strain levels. Our results are consistent with the hypothesis that there may be a strain threshold, which is required to activate the intracellular transduction pathways that generate the cell response. However, the mechanism for the regulation of PDGF-B expression by cyclic strain is still unknown. Previous studies in our laboratory have demonstrated that cyclic strain alters the second-messenger metabolic pathways, which may potentially play a role in the regulation of PDGF. For example, cyclic strain leads to activation of adenylate cyclase,<sup>43</sup> thereby increasing intracellular cAMP, which is also accompanied by an increase in protein kinase A activity. We have also reported

that exposure of ECs to cyclic strain stimulates production of diacylglycerol<sup>44</sup> and activates protein kinase C in a strain amplitude- and frequency-dependent manner.<sup>45-47</sup> This behavior leads to downstream activation of some but not all of the *fos* and *jun* family binding proteins.<sup>48</sup>

Activation of these "upstream" mediators in the cytosol is translated to nuclear events by induction of specific nuclear transcription factors. We have previously reported an increase in transcription factors AP-1, CRE, and NF- $\kappa$ B by EMSA of nuclear extracts from ECs subjected to cyclic strain.<sup>25</sup> We have now demonstrated by 5' deletion analysis (Fig 3B) that strain-inducible PDGF-B promoter-dependent expression is largely mediated by elements located in the region -313 to -153. Since the SSRE is located at position -125, these findings argue against a functional role for this element in the context of strain. Indeed, a heterologous promoter-reporter construct bearing the SSRE fails to respond to cyclic strain (Fig 5A). Moreover, mutation of the SSRE in the context of the -153 PDGF-B promoter-reporter construct fails to abolish strain-inducible gene expression (Fig 5B). These findings suggest that the SSRE, by itself, does not mediate strain-inducible PDGF-B expression. Instead, a functional role for elements upstream of the SSRE is implicated. Putative binding sites for Sp-1/Egr-1 and NF- $\kappa$ B are located at bp -21 and -180, respectively,<sup>21-22</sup> but further studies will need to be performed to determine whether these factors are involved. Nevertheless, the present study clearly suggests that strain-inducible PDGF-B expression may involve cooperativity between factors interacting with these upstream regions and those binding to the SSRE. This premise is consistent with the complexity of transcriptional control and the convergence and divergence of multiple signaling pathways. In this regard, recent studies by our group have demonstrated the important regulatory effect of NF- $\kappa$ B on the SSRE binding site.<sup>21</sup>

In conclusion, the present study demonstrates enhanced expression of PDGF-B in ECs exposed to cyclic strain. A minimal reporter sequence located 313 bp upstream of the start site is needed for maximal stimulation. Although induction of an SSRE binding protein has been demonstrated, the binding site at position -125 does not appear to be necessary for the strain-induced (unlike the shear-induced) activation of this gene. Further studies are needed to delineate the critical strain-induced binding sites in the PDGF-B promoter.

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### References

- Waterfield MD, Scragg GT, Whittle N, Stroobant P, Jonsson A, Wasteson A, Westermark B, Heldin C-H, Huang JS, Deuel TF. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature*. 1983;304:35-39.
- Siegbahn A, Hammacher A, Westermark B, Heldin C-H. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes and granulocytes. *J Clin Invest*. 1990;85:916-920.
- Sachinides A, Locher R, Vetter W, Tatje D, Hoppe J. Different effects of platelet derived growth factor isoforms on rat vascular smooth muscle cells. *J Biol Chem*. 1990;265:10238-10243.
- Heldin C-H, Ostman A, Westermark B. Structure of platelet-derived growth factor: implications for functional properties. *Growth Factors*. 1993; 8:245-252.
- Hart CE, Forstrom JW, Kell JD, Seifert RA, Smith RA, Ros R, Murray MJ, Bowen-Pope DF. Two classes of PDGF receptor recognize different isoforms of PDGF. *Science*. 1988;240:1529-1531.
- Ulich H, Li N, Frangos JA. Shear stress increases endothelial platelet-derived growth factor mRNA levels. *Am J Physiol*. 1991;260:H642-H646.
- Malek AM, Gibbons GH, Dzau VJ, Izumo S. Fluid shear stress differentially modulates expression of genes encoding basic fibroblast growth factor and platelet-derived growth factor B chain in vascular endothelium. *J Clin Invest*. 1993;92:2013-2021.
- Mitsumata M, Fische RS, Nerem RM, Alexander RW, Berk BC. Fluid shear stress stimulates platelet-derived growth factor expression in endothelial cells. *Am J Physiol*. 1993;265:H3-H8.
- Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF, Gimbrone MA. Platelet-derived growth factor B chain promoter contains a cis-acting shear stress-responsive element. *Proc Natl Acad Sci U S A*. 1993;90:4591-4595.
- Banes AJ, Gilbert J, Taylor D, Monbureau O. A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells in vitro. *J Cell Sci*. 1985;75:35-42.
- Sumpio BE, Banes AJ, Levin LG, Johnson G. Mechanical stress stimulates aortic endothelial cells to proliferate. *J Vasc Surg*. 1987;6:252-256.
- Sumpio BE, Banes AJ, Buckley M, Johnson G. Alterations in aortic endothelial cell morphology and cytoskeletal protein synthesis during cyclic tensional deformation. *J Vasc Surg*. 1988;7:130-138.
- Banes AJ, Link GW, Gilbert JW, Tay RTS, Monbureau O. Culturing cells in a mechanically active environment. *Am Biotech Lab*. 1990;8:12-22.
- Gilbert JA, Banes AJ, Link GW, Jones GJ. Surface strain of living cells in a mechanically active, in vitro environment. In: Dietrich DE, ed. *ANSIS Conference Proceedings*. Houston, Pa: Swanson Analysis Systems; 1989: 13.2-13.7.
- Gilbert JA, Banes AJ, Link GW, Jones GL. Video analysis of membrane strain: an application in cell stretching. *Exp Technique*. 1990;14:5:43-45.
- Iba T, Sumpio BE. Tissue plasminogen activator expression in endothelial cells exposed to cyclic strain in vitro. *Cell Transplant*. 1992;1:43-50.
- Li G, Mills I, Sumpio BE. Cyclic strain stimulates endothelial cell proliferation: characterization of strain requirements. *Endothelium*. 1994;22: 177-181.
- Awolosi MA, Sessa WC, Sumpio BE. Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. *J Clin Invest*. 1995;96: 1449-1454.
- Chirgwin JM, Macdonald PR, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 1978; 17:5294-5299.
- Greenberg ME, Ziff EB. Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. *Nature*. 1984;311:433-438.
- Khachigian LM, Resnick N, Gimbrone MA, Collins T. Nuclear factor- $\kappa$ B interacts functionally with the platelet-derived growth factor B-chain shear-stress-response-element in vascular endothelial cells exposed to fluid shear stress. *J Clin Invest*. 1995;96:1169-1175.
- Maniatis T, Fritsch EF, Sambrook J. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1982.
- Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian cells. *Nucleic Acids Res*. 1983;11:1475-1489.
- Glover DM. *DN4 Cloning*. Oxford, UK: IRL; 1985:213-238.
- Du W, Mills I, Sumpio BE. Cyclic strain causes heterogeneous induction of transcription factors, AP-1, CRE binding protein and NF- $\kappa$ B, in endothelial cells: species and vascular bed diversity. *J Biomech*. 1995;28: 1485-1491.
- Resnick N, Gimbrone MA. Hemodynamic forces are complex regulators of endothelial gene expression. *FASEB J*. 1995;9:874-882.
- Steinman DA, Ethier CR. The effect of wall distensibility on flow in a 2D end-to-side anastomosis. *J Biomech Eng*. 1994;116:294-301.
- Patel DJ, Greenfield JC, Austen WG, Morrow AG, Fry DL. Pressure-flow relationships in the ascending aorta and femoral artery of man. *J Appl Physiol*. 1965;20:459-463.
- Bergel DH. The static elastic properties of the arterial wall. *J Physiol (Lond)*. 1961;156:445-457.
- Awolosi MA, Sessa WC, Sumpio BE. Cyclic strain increases endothelial nitric oxide synthase. *Surgery*. 1994;116:439-444.

31. Sumpio BE, Banes AJ. Prostacyclin synthetic activity in cultured aortic endothelial cells undergoing cyclic mechanical deformation. *Surgery*. 1988; 104:383-389.
32. Sumpio BE, Widmann MD. Enhanced production of an endothelium derived contracting factor by endothelial cells subjected to pulsatile stretch. *Surgery*. 1990;108:277-282.
33. Iba T, Sumpio BE. Morphologic evaluation of human endothelial cells subjected to repetitive stretch in vitro. *Microvasc Res*. 1991;42:245-254.
34. Yano Y, Geibel J, Sumpio B. Tyrosine phosphorylation of pp125FAK and paxillin in aortic endothelial cells induced by mechanical strain. *Am J Physiol*. 1996;271:C635-C649.
35. Yano Y, Saito Y, Narumiya S, Sumpio B. Involvement of rho p21 in cyclic strain-induced tyrosine phosphorylation of focal adhesion kinase (pp125FAK), morphological changes and migration of endothelial cells. *Biochem Biophys Res Commun*. 1996;224:508-515.
36. Ranjan V, Xiao Z, Diamond SL. Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. *Am J Physiol*. 1995;269: H550-H555.
37. Shyy Y, Hsieh H, Usami S, Chien S. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci U S A*. 1994;91:4678-4682.
38. Malek AM, Greene AL, Izumo S. Regulation of endothelin-1 gene by fluid shear stress is transcriptionally mediated and independent of protein kinase C and cAMP. *Proc Natl Acad Sci U S A*. 1993;90:5999-6003.
39. Nagel T, Resnick N, Atkinson WJ, Dewey CF, Gimbrone MA. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J Clin Invest*. 1994;94: 885-891.
40. Thubrikar MJ, Robicsek F. Pressure-induced arterial wall stress and atherosclerosis. *Ann Thorac Surg*. 1995;59:1594-1603.
41. Darrett TD, Benditt EP. Platelet-derived growth factor B chain gene transcript levels are elevated in human atherosclerotic lesions compared to normal arteries. *Proc Natl Acad Sci U S A*. 1987;84:1099-1103.
42. Sarzani R, Brecher P, Chobanian AV. Growth factor expression in aorta of normotensive and hypertensive rats. *J Clin Invest*. 1989;83:1404-1408.
43. Cohen CR, Mills I, Du W, Kamal K, Sumpio BE. Activation of adenylyl cyclase/cyclic AMP/protein kinase A pathway in endothelial cells exposed to cyclic strain. *Exp Cell Res*. 1997;231:234-245.
44. Evans L, Frenkel L, Brophy C, Rosales O, Sudhaker S, Li G, Du W, Sumpio B. Activation of diacylglycerol in cultured endothelial cells exposed to cyclic strain. *Am J Physiol*. 1997;272:C123-C130.
45. Brophy C, Mills I, Rosales O, Isales C, Sumpio B. Phospholipase C: a putative mechanotransducer for endothelial cell response to acute hemodynamic changes. *Biochem Biophys Res Commun*. 1993;190:576-581.
46. Rosales O, Sumpio BE. Changes in cyclic strain increase inositol triphosphate and diacylglycerol in endothelial cells. *Am J Physiol*. 1992;262: C956-C962.
47. Rosales O, Sumpio BE. Protein kinase C is a mediator of the adaptation of vascular endothelial cells to cyclic strain in vitro. *Surgery*. 1992;112: 459-466.
48. Sumpio BE, Du W, Xu W. Exposure of endothelial cells to cyclic strain induces c-fos, fosB and c-jun but not junB or junD and increases the transcription factor AP-1. *Endothelium*. 1994;22:149-156.
49. Khachigian LM, Fries JW, Benz MW, Bonchrou DT, Collins T. Novel *cis*-acting elements in the human platelet-derived growth factor B-chain core promoter that mediate gene expression in cultured vascular endothelial cells. *J Biol Chem*. 1995;269:22647-22656.